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A Novel Approach for the Identification of Unique Tumor Vasculature Binding Peptides Using an *E. coli* Peptide Display Library

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Background: Tumor neovascularization is necessary for continued tumor growth and metastasis. During the process of endothelial cell (EC) recruitment and tumor infiltration, specific molecular markers unique for this interaction are expressed on the EC surface. Targeting these molecular markers would, in effect, allow for specific tumor targeting. Tripeptide sequence motifs have previously been reported that will bind to angiogenic tumor ECs. These sequences were identified from in vivo phage peptide display libraries. The purpose of this study was to use a more simplified bacterial peptide display library in an in vitro system to seek out peptide motifs with unique binding to tumor microvasculature.

Methods: FliTrx™ is a bacterial peptide display library containing the entire repertoire of possible random dodecapeptides expressed on the flagella tip of *E. coli*. Two EC populations were used for the screening process, Matrigel invading cells (MAGIC) and tumor-derived endothelial cells (TDEC). MAGIC are obtained from ECs that infiltrate a subcutaneous fibroblast growth factor-containing Matrigel deposit, and TDEC are ECs selectively obtained from tumor vasculature. FliTrx cells were incubated with MAGIC at 4°C to remove any potential clones displaying peptides that will bind to nonspecific EC surface targets. The non-binding cells were then incubated with TDEC, allowing for clones displaying potential binding peptides to bind tumor specific targets on TDECs. The bacterial population was then expanded and this "panning" process was carried out a total of five times. Peptide insert sequences from 100 bacterial colonies were analyzed for potential repetitive peptide motifs.

Results: Recurring peptide sequences were detected that were 3-mers (13 sequences) and 4-mers (4 sequences). Of the 3-mers, four repeated 3 times, whereas none of the 4-mers repeated more than twice. All of the repeated sequences were basic in charge, and arginine was the most commonly seen amino acid. A tripeptide basic-basic-nonpolar amino acid arrangement was the most prevalent charge sequence in all repetitive motifs (17 repeat sequences). Two test peptides showed TDEC binding specificity, and both conformed to the basic-basic-nonpolar motif.

Conclusions: We report peptide sequences derived from panning an in vitro system designed to detect tumor-EC specific markers. These putative motifs may serve as molecular determinants for a novel therapeutic modality aimed at specifically targeting tumors through tumor angiogenic vessels.

Key Words: Angiogenesis—Tumor endothelium—Peptide display library—Tumor targeting.

Vascular endothelial cells cover the entire inner surface of blood vessels in the body. They play an important role in tissue homeostasis, fibrinolysis, coagulation, blood-tissue exchange, vascularization of normal and neoplastic tissues, and blood cell activation and migration during physiological and pathological processes. A unique aspect of endothelial cells is that although they present many common functional and morphological features, they also display remarkable heterogeneity in different organs. It has been shown that bovine aortic endothelial cells, when co-cultured with cells from various organs, change their phenotypes to reflect their

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interaction with that particular tissue type.[1] These phenotypes are mediated, in part, by molecular markers that are expressed by these endothelial cells specific for the unique interaction. Based on the unique histologic appearance of tumor vasculature, it is postulated that expression of specific molecular endothelial markers probably also exists for the tumor-endothelial interaction. The ability to target these molecules would, in effect, target the tumor endothelium specifically, and hence also the tumor.

Until recently, identification of tissue-specific endothelial markers has progressed slowly, partly because of difficulties in isolating pure populations of endothelial cells from tissues. Moreover, isolated and cultured cells may lose their tissue-specific traits upon passage in vitro. Thus, the phenotype of endothelial cells is unstable and likely to change when the cells are removed from their microenvironment.

Phage-display peptide libraries have been used to obtain defined peptide sequences interacting with a particular molecule in in vivo models. In this system, peptides with as many as 10^9 permutations are expressed on the phage surface by fusion with a phage surface protein. The desired peptides are selected on the basis of binding to the target molecule. The strength of this technology is its ability to identify interactive proteins and other molecules without preexisting notions about the nature of the interaction. Using this methodology, Ruoslahti et al. have elegantly identified various peptides with binding specificity to angiogenic endothelium and endothelial cells from various organs.[2,3] These peptide sequences typically are three amino acids in length, and the best characterized of these sequences is represented by the RGD (Arg-Gly-Asp) motif. RGD is the cell attachment site for many adhesive proteins.[4–9] This sequence was discovered in fibronectin,[10] and later was identified using in vivo phage peptide display library animal experiments.

The surprising finding that only three amino acids would form an essential recognition site for cells in a very large protein was at first received with some skepticism. However, the observation was soon confirmed with regard to fibronectin and then extended to other proteins. Since then, other peptide motifs that identify and bind to specific targets on the endothelial cells of angiogenic vessels,[11–13] brain,[2,3] lungs,[3] retina,[3] and kidneys[3] have been reported (Fig. 1). Although in vivo screening with phage peptide display libraries is a powerful technique for identifying peptides with specific protein-protein binding capabilities, it remains a laborious procedure. In this work, we used a more simplified in vitro *E. coli* system (FliTrx *E. coli* peptide display library)[14,15] in identifying peptide sequences with specific molecular targeting to the tumor endothelium.

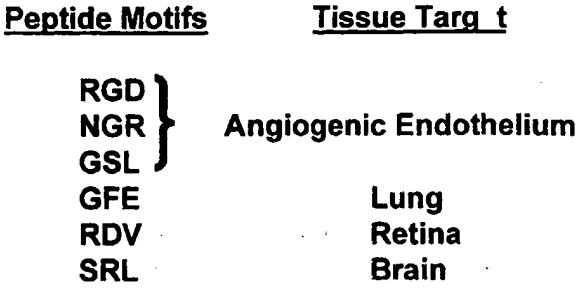


FIG. 1. Reported peptide motifs and their targeted tissue. These tripeptides have been shown in previous reports to target the designated tissues. RGD, Arg-Gly-Glu; NGR, Gln-Gly-Arg; GSL, Gly-Ser-Leu; GFE, Gly-Phe-Asp; RDV, Arg-Glu-Val; SRL, Ser-Arg-Leu.

MATERIALS AND METHODS

Panning Using FliTrx

We used a peptide display library, FliTrx™ (Invitrogen, Carlsbad, CA), in our efforts to identify peptides with tumor endothelial binding specificity. FliTrx is an *E. coli* system that expresses a random 12-amino-acid peptide on the flagella tip of the bacteria. Each organism expresses a specific peptide, and the collection of all organisms expressing all possible amino acid combinations constitutes the displayed peptide library. Each peptide sequence is flanked by cysteine residues, which form a disulfide loop that constrains the expressed peptide. This conformational constraint has been shown to improve binding of peptides to its target molecule.

Our goal was to identify peptides with binding specificity for tumor endothelium. We hoped to achieve this goal by initially panning the bacterial library against a monolayer of Matrigel infiltrating cells (MAGIC) to remove potential clones that will bind to nonspecific cellular markers. MAGICs are angiogenic endothelial cells that infiltrate a subcutaneous fibroblast growth factor and heparin-containing Matrigel® (Collaborative Biomedical Products, Bedford, MA) deposit.[16,17] These cells lack tumor cell interaction and presumably should not display any tumor specific markers. Conversely, tumor-derived endothelial cells (TDEC) are endothelial cells from a subcutaneous tumor xenograft. Following "subtraction-panning" with MAGICs, the non-binding clones are incubated with TDECs. Clones binding to TDECs are then propagated, which should amplify those organisms displaying peptides with tumor endothelial specificity. This process is repeated five times, each time removing clones binding to MAGIC while enriching for those binding to TDEC (Fig. 2).

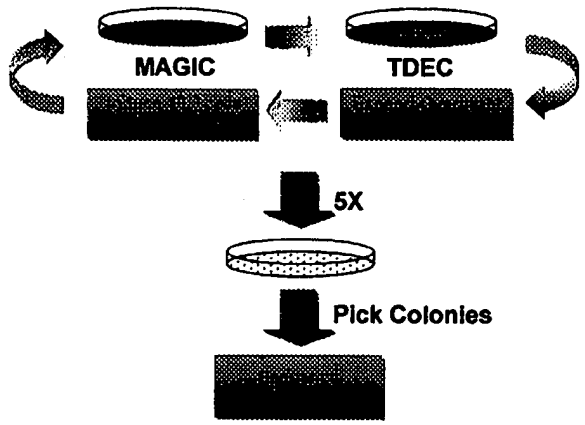


FIG. 2. General in vitro panning protocol using an *E. coli* peptide display library.

We used one vial of FliTrx Peptide Library to inoculate 50 ml of IMC medium [1 × M9 Salts (40 mM Na_2HPO_4, 20 mM KH_2PO_4, 8.5 mM NaCl, 20 mM NH_4Cl, pH 7.4), 0.2% casamino acids, 0.5% glucose, 1 mM $MgCl_2$] containing 100 μg/ml of ampicillin. The inoculated broths were grown overnight with shaking at 25°C until OD_{600} was approximately 3. To induce peptide expression by the *E. coli* cells, 10^{10} cells were added to 50 ml of IMC medium containing 100 μg/ml each of ampicillin and tryptophan and incubated at 25°C with shaking for 6 hours. After peptide induction, 10^{10} bacterial cells were layered on confluent MAGICs in a 100-mm dish. The incubation was performed at 4°C for 1 hour in the presence of 10% fetal calf serum (FCS) and 1% α-methyl mannoside. The presence of FCS prevented nonspecific protein-protein binding, whereas that of α-methyl mannoside prevented nonspecific protein binding via lectin-like interactions. After the 1-hour period, the non-binding cells were transferred directly onto TDECs and incubated under conditions similar to those used with MAGICs. The TDEC monolayer was washed five times with IMC medium containing 10% FCS and 1% α-methyl mannoside (wash solution) to remove non-binding cells. Cells bound to TDECs were dislodged by vigorously vortexing the plate containing 0.75 ml of wash solution for 30 seconds. These cells were then amplified and reinduced for peptide expression, and the process of panning was repeated four additional times.

Generation of TDEC and MAGIC

TDECs are endothelial cells from SCC VII murine squamous cell tumors in C3H/HeJ mice[18] (Fig. 3A). Approximately 10^6 SCC VII cells were injected subcutaneously into each C3H/HeJ mouse. After a tumor had achieved a diameter of 0.5 cm to 1.0 cm and before there was any evidence of central necrosis, it was removed sterilely, minced, and digested with collagenases and RNAses to yield a single cell suspension. The cells were then stained using antibody-recognizing platelet-endothelial cell adhesion molecules (PECAM). The stained cells were sorted using automated flow cytometry, and the positive cells were cultured as TDECs in Po Media (DMEM supplemented with 20% Sarcoma-180 conditioned media, 10% FCS, 1% BME vitamin, 1% heparin, and 0.005% endothelial cell growth supplement [Collaborative Research]).

MAGICs are obtained in a similar fashion (Fig. 3B). Approximately 7 to 10 days after the Matrigel injection, the plug was removed and cultured in vitro in Po Media for 7 to 10 more days. Single cell suspension was prepared by digesting the Matrigel plug in Dispase® (Collaborative Biomedical Products, Bedford, MA). The cells were then cultured in Po Media.

In Vitro Binding of Peptides With TDEC and NIH3T3

Evidence for specific oligopeptide binding to TDECs was acquired as follows. The most frequently repeated sequences from the 100 clones were used for synthesis of oligopeptides (Sigma Genesis). By conjugating fluorescein FITC to the carboxyl termini of peptides, these molecules were tested for in vitro binding with TDECs and NIH3T3 cells (negative control cell line). These peptides contain flanking cysteines so that a disulfide loop may be reformed, thus constraining and mimicking the binding condition of the initial selection process. Positive and negative control peptides were poly-L-lysine oligopeptides (Sigma) and glycine heptamers ($PepG_7$), respectively. $PepG_7$ is not expected to have any specific binding to membrane proteins, whereas poly-L-lysine is a

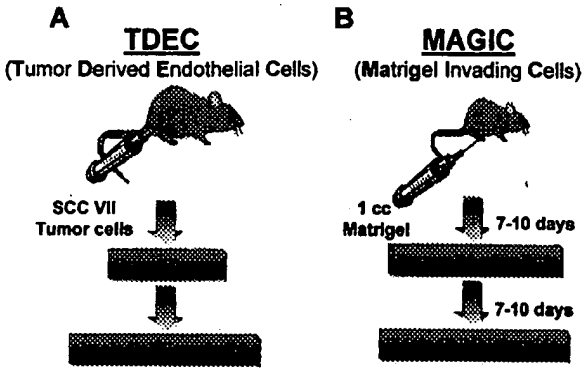


FIG. 3. Isolation of (A) tumor-derived endothelial cells (TDEC) and (B) MatriGel invading cells (MAGIC).

nonspecific cell binder, and therefore, should bind both NIH3T3 and TDECs. We incubated monolayers of NIH3T3 and TDECs on glass chamber slides with peptides at 5 μg/ml (1 μg of peptide per 200 μl well) overnight at 37°C in phosphate-buffered saline (PBS) with 10% FCS. Prior to binding, monolayers were flash-fixed with 3.7% (w/v) formamide at room temperature for 10 minutes. The cells were washed three times with PBS containing 10% FCS at 37°C for 15 minutes, and fluorescent microscopy then was performed.

RESULTS

Oligopeptide Sequences Obtained From Panning Experiments

Following panning of the FliTrx library against MAGIC and TDEC, 100 colonies were picked randomly for insert analysis and resulted in 95 interpretable peptide sequences. The resulting sequences were then analyzed for repeats. Those repeated sequences (3-mer or greater) are listed in Fig. 4. A total of 17 repeat sequences were isolated—thirteen 3-mers and four 4-mers. From these sequences, no single peptide sequence was predominant. However, when the peptide sequences were further analyzed according to the chemical properties of each amino acid, a pattern did emerge. The individual amino acids were scored as either positively charged (+), negatively charged (−), neutral and hydrophilic (⊘), or neutral and hydrophobic (○) in physiologic conditions. This analysis revealed that of all the repeats, seventeen clones depicted a positive-positive-hydrophobic motif (++○) (Fig. 5).

Arg-Arg-Val-Leu	2
Arg-Arg-His-Glu	2
Arg-Arg-Ser	**3**
Arg-Arg-Leu	2
Arg-His-Ser	2
Gly-Arg-His-Ser	2
Gly-Arg-His	**3**
Arg-Lys-Leu	2
Arg-Lys-Ile	2
His-Lys-Ile	2
Lys-Arg-Ala	**3**
Ser-Lys-Arg-Ala	2
Arg-Ser-Arg	**3**
Arg-Ser-Trp	2
Ser-Arg-Ala	2
Ser-Arg-Gly	2
Arg-Gly-His	2

Size / #	3-mer	4-mer
2X	9	4
3X	4	0

FIG. 4. Sequences and frequency of the repeated peptides. Insert sequences of the displayed peptides from 100 randomly picked clones were analyzed for three- or four-amino-acid sequences. The oligopeptide sequences that occurred in more than one clone are depicted along with their frequency of occurrence. The table summarizes the result. There were nine 3-mers that repeated twice, four 4-mers that repeated twice, and four 3-mers that repeated three times.

Charge Distribution of Peptide Sequences

++○	17
++⊘	9
⊘++	8
+⊘+	5
⊘++⊘	3
⊘++○	2
○++○	2
++○○	2
○++	2
+++	2
⊘+⊘	2
⊘+○	2
+⊘○	2

FIG. 5. Frequency of peptide repeats based on the chemical properties of the amino acids. The peptide repeats were analyzed for the charge characteristics of its amino acids at physiologic pH. (+, basic at physiologic pH; −, acidic at physiologic pH; ○, neutral and hydrophobic at physiologic pH; ⊘, neutral and hydrophilic at physiologic pH).

Combining the data from Figs. 4 and 5, we derived five peptide sequences to be tested for TDEC binding specificity (Fig.6). The test peptides are labeled PepMW1 to PepMW5. $PepG_7$ consists of seven glycine residues flanked by cysteines. This peptide should have no binding specificity to TDEC and serves as the negative control peptide. Poly-L-lysine is a nonspecific cellular binding peptide and serves as the positive control peptide for in vitro binding experiments.

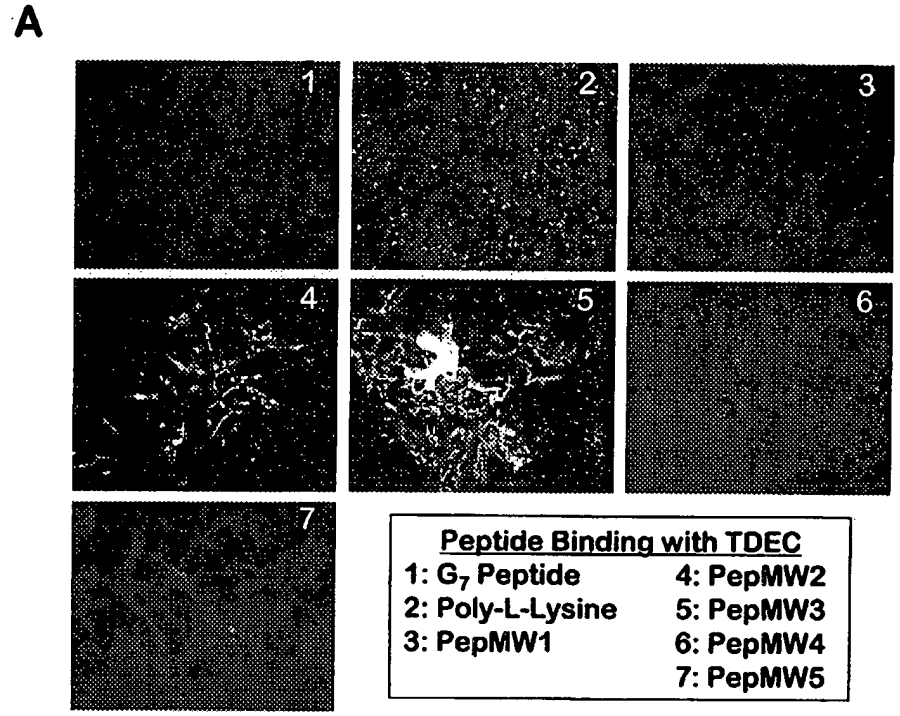
Test Peptide Sequences

PepMW1	**C-G-G-Arg-His-Ser-G-G-C**
PepMW2	**C-G-G-Arg-Lys-Leu-G-G-C**
PepMW3	**C-G-G-Arg-Arg-Leu-G-G-C**
PepMW4	**C-G-G-Arg-Arg-Ser-Arg-G-G-C**
PepMW5	**C-L-L-Arg-Arg-Ser-Arg-L-L-C**
$PepG_7$	**$C\text{-}G_7\text{-}C$**
Poly-L-Lysine	**$(\text{-Lys-})_n$**

FIG. 6. Sequences of test peptides. Sequences of peptides to be tested for binding specificity to TDEC are depicted. Each peptide is flanked by cysteine residues to allow for disulfide linkage and loop formation. Glycine residues provide a "spacer" function, permitting the physical formation of a loop structure. The glycines in PepMW4 are replaced by leucines in PepMW5. $PepG_7$ serves as the negative control peptide, whereas poly-L-lysine serves as the positive control peptide. Each peptide also is labeled at the carboxyl terminus with FITC for fluorescent microscopy.

In Vitro Binding of Peptide With TDEC

To determine the endothelial specificity of peptides PepMW1–5, we initially carried out in vitro binding experiments with TDEC monolayers. The results of this binding experiment are depicted in Fig. 7A. In these experiments, NIH3T3 was used as the negative control



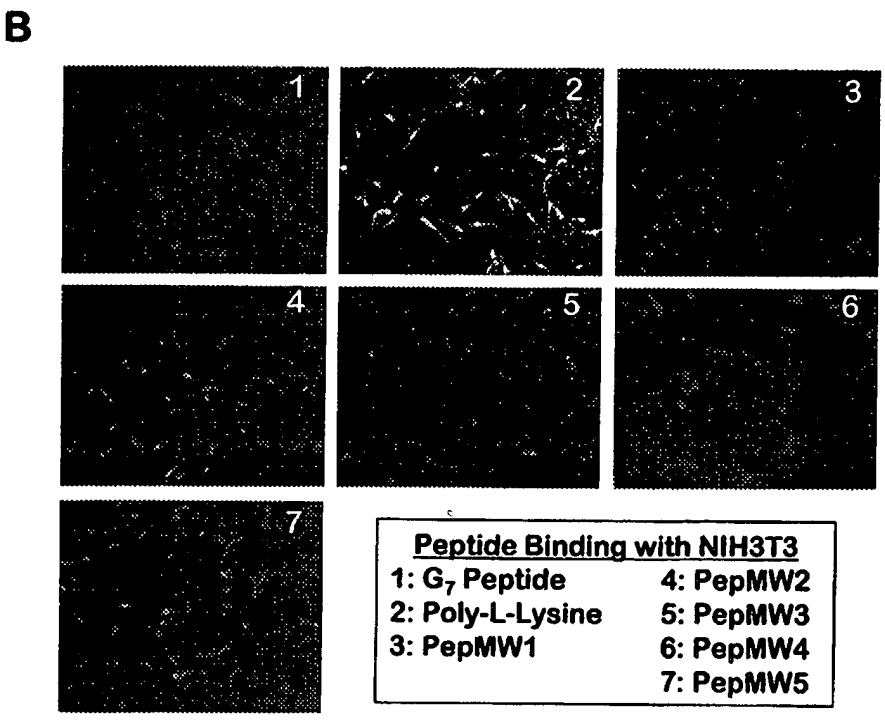


FIG. 7. In vitro binding of FITC conjugated peptides to TDEC. Binding of the respective peptides with TDEC (A) and NIH3T3 cells (B) are depicted. The peptides are incubated with TDEC monolayers cultured on glass slide wells. One μg of each peptide is added per 200-μl well. Incubation is performed at 37°C overnight. The monolayers were washed three times prior to fluorescent microscopy.

monolayer, and all peptides tested showed equal background binding except for poly-L-lysine (Fig. 7B). As can be seen in Fig.7, FITC-labeled PepMW3 (Arg-Arg-Leu) showed the brightest staining and specificity for TDEC. PepMW2 (Arg-Lys-Leu) also showed preferential staining for TDEC. However, binding of FITC-labeled PepMW2 to TDEC did not yield as high a level as did binding of PepMW3 to TDEC. Incidentally, both PepMW3 and PepMW2 conform to the positive-positive-hydrophobic (++○) motif. The replacement of the glycine residues with leucines in PepMW5 did not appear to have any change in binding of the peptide to TDEC. Both PepMW4 and PepMW5 exhibited background binding.

DISCUSSION

In this report, we describe an in vitro bacterial panning procedure for selection of peptides with tumor endothelial binding specificity. This is different from previously reported in vivo phage display library panning strategies, which have yielded peptide sequence motifs with tumor and tissue specificities. Each system has inherent advantages and disadvantages. One advantage of using a bacterial peptide display library over a phage peptide display library is its relative ease of use over the phage system. However, using a bacterial library precludes in vivo screening due to splenic and hepatic trapping of bacterial organisms.

The advantage of in vitro screening is that the screening conditions can be varied. Additionally, the selection markers can be specifically targeted by panning against specific cells or bound protein molecules. This stringent control over panning condition and selection markers is difficult to achieve in an in vivo screening process. Thus, the applications of in vitro systems are potentially wider in scope than those of in vivo systems.

One disadvantage of in vitro panning of cellular markers is its requirement for cultured cells. A concern is that cultured cells may not adequately reflect the true nature of their counterpart cells in vivo. In our case, TDECs may lose cell surface tumor-specific markers in culture due to lack of tumor-endothelial interaction. We believe that this interaction is the "driving pressure" maintaining the presence of tumor-specific molecular markers on endothelial cells. In an attempt to minimize this effect, we have limited in vitro passage of TDECs to a maximum of five times for the panning experiments.

The importance of pathologic angiogenesis is well established in the clinical setting of cancer. Solid tumors are not able to grow much larger than 2 mm in diameter without a blood supply, and to express a malignant phenotype, tumors must induce new vessel growth. Tumors recruit endothelial cells during the process of angiogenesis. The recruited endothelial cells differentiate and express unique molecular markers specific for their association with tumor cells. The ability to identify and target these molecular markers would allow for specific targeting of the tumor vasculature, and, therefore, the tumor as well. This creates the possibility of directing therapeutic modalities at the endothelial cells.

There is good rationale for therapeutic targeting of tumor endothelium rather than tumor cells themselves. Endothelial cells are nontransformed cells. The genetic information of cancer cells is inherently unstable, and tumor cells can mutate and develop resistance to a previously responsive therapeutic modality much faster than a genetically stable population of cells such as "normal" endothelial cells. Peptide molecules that are able to bind to tumor endothelium specifically could be used as a delivery vehicle for carrying cytotoxic agents to the tumor. The selective increased concentration of the cytotoxic agent within the tumor would affect not only the endothelial cells but also the tumor cells. Mortality due to cancer is the result of uninhibited and metastatic growth of the cancer made possible by the tumor vasculature. Therefore, the ability to destroy tumor vasculature leading to deterred cancer growth and metastasis would affect significantly the natural outcome of this disease process.

Aside from therapeutic targeting of the tumor vasculature, peptides that will specifically bind to tumor endothelium can be used as a possible diagnostic tool. We often encounter patients in our repertoire of clinical experience who have evidence of tumor, but disease cannot be localized. Potentially, these peptide molecules can be conjugated to radiologically "detectable" molecules and used to enhance imaging studies (i.e., computed tomography or magnetic resonance imaging scans) in such patients. Additionally, by using the peptides, molecular targets that are bound by the peptides can be isolated and identified. These molecular targets can then be used as potential tumor specific antigens for tumor immunotherapeutics. Furthermore, these molecular targets can also be used as potential tumor markers in patients' sera for the molecular detection of cancer cells. Thus, the identification of peptide motifs that will bind specifically to tumor endothelium has many therapeutic and diagnostic implications.

CONCLUSION

From the in vitro panning experiments, recurring peptides sequences were detected that are 3-mers (13 se-

quences) and 4-mers (4 sequences). Most of the detected sequences are basic, and arginine is the most common amino acid. Although no single peptide sequence stood out as the predominant repeat, analysis of the amino acid charge composition did reveal a positive-positive-hydrophobic (++O) motif predominating in the repeat peptide sequences. PepMW3 (Arg-Arg-Leu) appears to have the highest in vitro binding specificity to TDEC, and PepMW2 (Arg-Lys-Leu) also exhibits some binding specificity to TDEC. Incidentally, both PepMW3 and PepMW2 conform to the positive-positive-hydrophobic motif. Overall, it appears that in vitro panning using an. *E. coli* random peptide display library is a useful technique for the identification of peptides with specific protein-protein binding capabilities.

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